# Identification of Residues Involved in Ligand Binding to the Neurokinin-2 Receptor

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ABSTRACT: Several residues of the human neurokinin-2 receptor have been identified to be critical for the binding of peptide agonists and non-peptide antagonists. Amino acid substitutions in the first and second extracellular segments and the second transmembrane segment led to substantial reduction in peptide affinity without affecting the affinity of antagonist SR48968. These effects are identical to those observed for homologous residues in the neurokinin-1 receptor, suggesting that these three regions are involved in high-affinity peptide binding to both receptor subtypes. On the other hand, some conserved residues in the fourth to seventh transmembrane segments are required for peptide binding to only one receptor subtype but not both. The conserved nature and location of these receptor residues suggest that the distance between bound peptide and helices 4−7 varies depending on the receptor subtype. It is likely that the conformational compatibility between a ligand and a given receptor determines the magnitude of binding affinity, and thus receptor subtype selectivity. While many single-residue substitutions did not affect the binding affinity of the antagonist SR48968, two double mutants in the sixth and seventh transmembrane segments were found to reduce its affinity substantially. Therefore, receptor residues participate cooperatively in the binding of SR48968. These results demonstrate the usefulness of combining single-residue substitutions in studying and confirming the role of receptor residues in ligand binding. Finally, the overlapping nature of agonist and antagonist binding sites is consistent with the observation that substitutions of some residues modify the binding affinities of both peptide agonists and non-peptide antagonists.

Elucidating the molecular basis of ligand binding specificity remains an important aspect of receptor research. This issue can be illustrated by the three subtypes of neurokinin receptors (Helke et al., 1990; Iversen et al., 1987; Nakanishi, 1991), all of which bind the mammalian neuropeptides substance P (SP),[1] neurokinin A (NKA), and neurokinin B (NKB). The rank order of potency for the neurokinin-1 receptor (NK1R) is SP > NKA > NKB, while it is NKA > NKB > SP for the neurokinin-2 receptor (NK2R) and NKB > NKA > SP for the neurokinin-3 receptor (NK3R). Structure−activity studies on peptide ligands have identified the most critical residues on SP and NKA; some of these residues are unique to one particular peptide (Regoli et al., 1984, 1990). Because all three peptides contain the conserved C-terminal sequence FXGLM-$NH_2$, an address−message model has been proposed envisioning the C-terminal half as message (activity unit) and the N-terminal half as address (specificity unit). However, this model appears to be oversimplified when considered in the context of receptor interactions and does not explain the results of many recent mutagenesis studies (Fong et al., 1992; Fong & Strader, 1994; Gether et al., 1993; Sachais & Krause, 1994; Strader et al., 1994; Yokota et al., 1992). For example, site-directed substitutions of divergent residues of the NK1R reduce or increase the binding affinity for both intact peptides and C-terminal fragments, suggesting a lack of a corresponding specificity region in the receptor (Huang et al., 1994a; Werge, 1994). In a few cases where substituting a receptor residue with the corresponding residue from another receptor subtype produces a selective increase in the affinity of one peptide, the magnitude of the change is small (Fong et al., 1992; Huang et al., 1994b). Furthermore, some C-terminal hexapeptide analogs of SP are still selective for the NK1R, indicating that the specificity information can be coded in the conserved C-terminal portion of the peptide (Cascieri et al., 1992; Wormser et al., 1986).

A ligand binding site is defined here as the total receptor area that will be occupied upon ligand binding. Therefore, multiple receptor residues would be expected to constitute the ligand binding site. Previous studies of the NK1R have identified several residues that are prime candidates as part of the peptide binding site, including residues located at the first and second extracellular segments (abbreviated as E1 and E2) and the second, fourth, and seventh transmembrane segments (abbreviated as H2, H4, and H7) (Fong et al., 1994; Huang et al., 1994a; Rosenkilde et al., 1994). To elucidate the molecular determinants of receptor subtype specificity, it will be necessary to determine whether the NK2R utilizes similar residues as the NK1R for the binding of peptides. In the present report, we have analyzed the role of NK2R residues in ligand binding and receptor activation. Comparison of NK1R data and NK2R data indicate that many conserved residues in the E1 and E2 segments and the H2 segment are required for peptide binding in both receptors. Other conserved residues in the H4, H5, H6, and H7 segments may or may not be required for peptide binding depending on receptor subtype. Amino acid identity itself is not a major factor because the majority of critical residues are conserved, and amino acid substitutions at those positions affect the binding affinity of all three natural peptides. These

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[1] [$^{125}$I]-NKA, [$^{125}$I-His$^1$]neurokinin A; NK1R, neurokinin-1 receptor; NK2R, neurokinin-2 receptor; NKA, neurokinin A; NKB, neurokinin B; PI, phosphatidylinositol; SP, substance P.

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data suggest that local conformation in the H4, H5, H6, and H7 regions may be important for differentiating the NK1R binding pocket from the NK2R binding pocket. For a given receptor, it appears that the conformational compatibility of the ligand and the receptor plays a major role in generating the peptide affinity rank order. The present study also provides evidence for a dynamic interaction between the NK2R and the antagonist SR48968 involving residues in H6 and H7.

## MATERIALS AND METHODS

The human NK2R cDNA was cloned into the pCDM9 vector as described (Gerard et al., 1990; Huang et al., 1994a). All mutations were constructed from the human NK2R by the uracil selection method of site-directed mutagenesis (Bio-Rad, Richmond, CA). All mutated sequences were confirmed by DNA sequencing. All receptors were expressed in COS cells for determination of ligand binding affinity and receptor activation (Huang et al., 1994a). Intact cells were dissociated non-enzymatically (Specialty Media, Lavallette, NJ) for assays using [$^{125}$I]NKA (New England Nuclear, Boston, MA) or [$^{3}$H]SR48968 (Amersham, Arlington Heights, IL).

The binding affinities of various ligands for the wild-type and mutant human NK2Rs were determined using radioactive ligand and intact COS cells in suspension in the presence of varying concentrations of unlabeled ligands (Huang et al., 1994a). Briefly, the binding reaction mixture contained radiolabeled ligand (0.5 nM for [$^{125}$I]NKA or [$^{3}$H]SR48968), unlabeled ligands at various concentrations, and intact COS cells expressing the wild-type or the mutant receptor in 0.25 mL (for [$^{125}$I]NKA) or 0.5 mL (for [$^{3}$H]SR48968) of 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM $MnCl_2$, 0.04 mg/mL bacitracin, 0.004 mg/mL leupeptin, 0.2 mg/mL bovine serum albumin, and 0.01 mM phosphoramidon. The receptor concentration in the binding mixture was adjusted so that the bound counts per minute in the absence of unlabeled ligand was less than 10% of totally added radioligand. Binding was performed at 23 °C for 1 h and terminated by filtration through GF/C paper. The data were fitted to the equation (cpm[L] − cpm(1 $\mu$M NKA or SR48968))/(cpm(0) − cpm(1 $\mu$M NKA or SR48968)) = $IC_{50}$/([L] + $IC_{50}$), in which cpm([L]) and cpm(0) represent bound radiolabeled ligand in the presence or the absence of unlabeled ligand, respectively; [L] represents the concentration of unlabeled ligand; and $IC_{50}$ represents the concentration of unlabeled ligand that causes 50% inhibition of the specifically bound radiolabeled ligand. The receptor concentration and $K_i$ value are calculated as described (DeBlasi et al., 1989; Swillens, 1992). For NKA with [$^{125}$I]NKA or SR48968 with [$^{3}$H]SR48968, $K_i$ = $IC_{50}$ − [labeled ligand]. For unlabeled ligands with nonhomologous labeled ligand, $K_i$ = $IC_{50}$/(1 + [labeled ligand] / $K_i$ of labeled ligand).

The binding of [$^{125}$I]NKA to some receptor mutants was also tested using cells attached in monolayer (Rosenkilde et al., 1994). Because the same results were obtained using either a cell suspension or a monolayer of cells, all data presented in the paper were derived from cell suspension binding assay.

The formation of total inositol phosphates (inositol mono-, bis-, and trisphosphates) was measured essentially as described previously (Huang et al., 1994a). Briefly, $10^7$ COS cells were electroporated in the presence of 10 $\mu$g of the plasmid DNA and evenly distributed into 12-well plates at about 50 000 cells per well. Each well also contained 10 $\mu$L of [$^{3}$H]inositol (1 mCi/mL, 24.4 Ci/mmol). Two days after the transfection, each well was washed twice with PBS containing 10 mM LiCl, and LiCl was loaded into cells by incubation in the washing buffer at 37 °C for 30 min. Fresh washing buffer (1 mL) containing agonist (or agonist plus antagonist) at various concentrations was added to each well and incubated at 37 °C for 30 min. The reaction was stopped by removing the buffer, adding 500 $\mu$L of ice-cold 5% TCA, and allowing the mixture to stand at 4 °C for 10 min. The solubilization mixture was applied to a column containing Dowex AG1-X8 (formate form), washed with 10 mL of 5 mM inositol. Total inositol phosphates were eluted with 4 mL of 1 M ammonium formate−0.1 M formic acid.

The synthesis of non-peptide antagonists SR48968 and GR158897 has been described by others (Cooper et al., 1994; Hale et al., 1993). All peptides were purchased from Peninsula Laboratories.

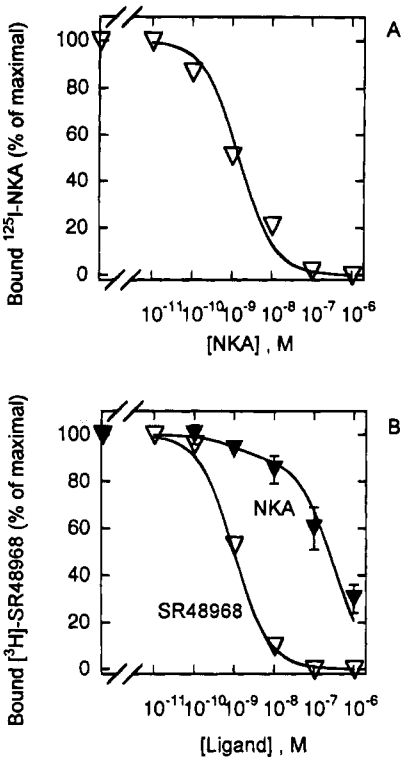


FIGURE 1: Inhibition of radiolabeled ligand binding to the wild-type NK2R by unlabeled ligands. (A) Total [$^{125}$I]NKA added was 239 000 cpm, of which 5100 cpm was specifically bound. The amount of high affinity state receptor was calculated to be 14 fmol/50 000 cells (Swillens, 1992). Data shown are representative of three independent experiments. (B) Total [$^{3}$H]SR48968 added was 15800 cpm, of which 900 cpm was specifically bound. The total amount of receptor was calculated to be 105 fmol/50 000 cells. Data shown are representative of five independent experiments. The solid line through the closed triangles is based on the simulation of a two-state model, with a high affinity of 1 nM (10%) and a low affinity of 300 nM (90%).

## RESULTS

*Determination of Peptide Binding Affinity Using Radiolabeled Antagonist.* The $IC_{50}$ value of NKA for the human NK2R determined using [$^{3}$H]SR48968 is higher than that using [$^{125}$I]NKA [Figure 1; see also Rosenkilde et al. (1994)].

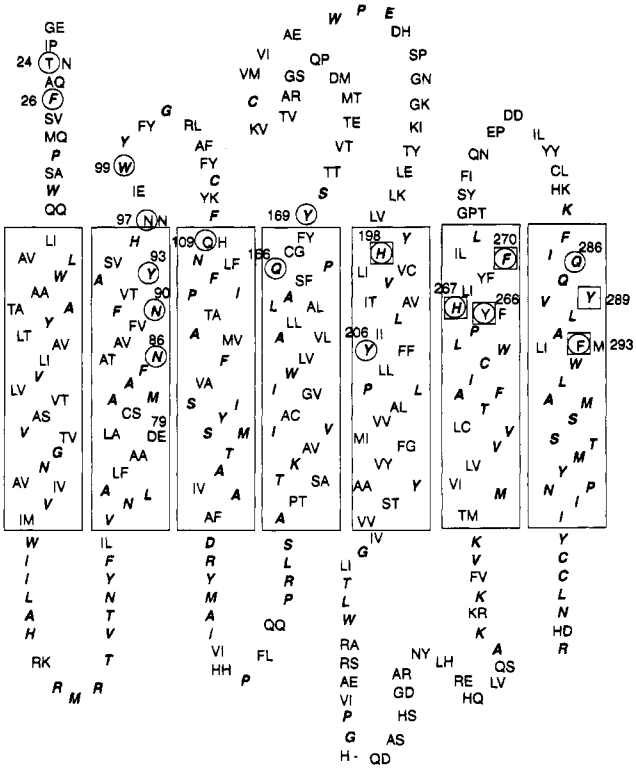

FIGURE 2: Schematic model of the human NK2R and NK1R. Bold italic letters represent residues conserved among all three subtypes of NK receptors. The NK2R residues are shown at left, and the NK1R residues are shown at right. The numbering is based on the NK2R sequence. The circled residues are postulated to form part of the peptide binding site, and the boxed residues are postulated to interact with at least one non-peptide antagonist.

Two factors contribute to the difference. When using [$^{125}$I]-NKA at 0.5 nM, the radioligand will bind to receptors if the affinity is less than about 10 nM, and thus the $K_i$ determined for NKA reflects its binding to the high-affinity state (i.e., G protein associated). In addition, [$^3$H]SR48968 has the same affinity for the NK2R regardless of G protein association. Therefore, the $K_i$ of NKA measured against [$^3$H]-SR48968 should fall between the intrinsic affinities of NKA for the low-affinity and high-affinity states, depending on the ratio of the two receptor populations. When the total receptor concentration was estimated from [$^3$H]SR48968 binding (Figure 1B) and compared to the concentration of receptor in the high-affinity state (Figure 1A), the high-affinity NK2R accounted for 14% of all NK2R. Under these conditions, the $K_i$ derived from experiments such as those in Figure 1B would approximate the affinity of NKA for the low-affinity state of NK2R.

*Effects of Amino Acid Substitutions on NKA Binding.* To determine which residues are required for NKA binding, a selected residue (e.g., Asn-86) was substituted with a smaller aliphatic residue such as Ala or Leu (Figure 2). When using 50 000−100 000 cells in suspension in the binding assay, the N86A substitution resulted in a very low level of [$^{125}$I]-NKA binding (1000 cpm or less) compared to the wild-type receptor (10 000−20 000 cpm). Increasing the cell number 2−3-folds did not lead to increased specific binding of [$^{125}$I]-NKA for the mutant receptor. On the other hand, the same level of [$^3$H]SR48968 binding was observed for both the wild-type and the N86A mutant under identical conditions with the same number of cells, and the binding affinity of SR48968 was not affected by the N86A substitution (Table1). The very low level of [$^{125}$I]NKA binding was also observed when a monolayer of cells was used in the binding assay as described by Rosenkilde et al. (1994). Both agonist and antagonist binding suggested that the NKA affinity was reduced for the N86A mutant. Many mutant receptors in the present paper exhibited properties similar to those of the N86A mutant. Therefore, [$^3$H]SR48968 was used for all mutant receptors to estimate the NKA binding affinity for the low-affinity state of the NK2R when a mutant receptor exhibited a very low level of [$^{125}$I]NKA binding, and a functional assay was used to estimate the NKA affinity for the high-affinity state of the NK2R.

Along with the N86A mutation, other residues throughout the extracellular and transmembrane regions of the human NK2R were analyzed (Table 1). Many of the residues were selected because the homologous residues in the NK1R have been found to be required for peptide binding (Huang et al., 1994a). Many mutant receptors in Table 1 had substantially reduced affinity for NKA, as indicated by the undetectable level of [$^{125}$I]NKA binding. Therefore, the peptide binding affinity for the low-affinity state of the NK2R was measured by the inhibition of [$^3$H]SR48968 binding. These studies confirmed the reduced affinity of NKA for the mutant receptors compared to the wild-type receptor. Furthermore, when a substitution resulted in reduced affinity of NKA, the rank order of potency for peptides was not changed (Table 1). An example is shown in Figure 3 for the N86A mutant. On the other hand, none of the single-residue substitutions (except W99A and Y289F) affected the binding affinity of SR48968 and the expression level of the NK2R as measured by [$^3$H]SR48968 binding, suggesting that these substitutions affect peptide binding specifically. In addition, these mutant receptors can be activated by agonist (see below), indicating that these residues are not required for activation. Of all substitutions tested so far, S27A, W31A, F89A, Q109A, F112A, T115A, S170A, S274, and H282A did not substantially affect the binding affinity of either NKA or SR48968 using either [$^3$H]SR48968 or [$^{125}$I]NKA (Table 1).

*Effects of Amino Acid Substitutions on Receptor Activation.* When the ability of various mutant receptors to activate the second messenger pathway was tested, most substitution mutants shown in Table 1 were still capable of stimulating phosphatidylinositol hydrolysis. These mutant receptors also displayed a dose−response curve that was shifted to the right compared to the wild-type receptor, consistent with a reduced affinity of NKA for the high-affinity state of the NK2R. In contrast, the D79A mutant was inactive in the presence of agonists at 10 $\mu$M. These data suggest that Asp-79 may be involved in receptor activation.

When the binding of agonists is measured, changes in the receptor activation process will also affect the apparent binding affinity (Colquhoun & Farrant, 1993). Since the activation step in G protein coupled receptors cannot be determined independently, this issue can only be addressed qualitatively. When the potency of NKA in stimulating a functional response ($EC_{50}$) was compared to its ability to inhibit the binding of [$^3$H]SR48968 ($K_i$), the two values were correlated among several mutant receptors and the wild-type receptor (Figure 4). These results are consistent with the interpretation that modifications at these positions primarily affect peptide binding. However, three substitutions seem to increase the $EC_{50}$ value more than the apparent $K_i$ value

Table 1: Effect of Amino Acid Substitution on Agonist or Antagonist Binding and on Receptor Activation

| | $K_i$, (nM)[a] | | | | PI hydrolysis in response to NKA[b] | |
|---|---|---|---|---|---|---|
| receptor | NKA | NKB | SP | SR48968 | $EC_{50}$, nM | maximal response @10 $\mu$M |
| NK2R | 130 (6) or 1.0* (3) | 6000 | >10000 | 0.7 (5) or 0.1 * (3) | 2 (3) | 100% |
| T24A | >10000 | >10000 | >10000 | 0.4 (2) | >1000 | 67% |
| F26A | >10000 | >10000 | >10000 | 1.5 (2) | >1000 | 33% |
| S27A | 0.2 * (2) | ND | ND | 0.6 * (2) | ND | ND |
| W31A | 0.2 * (2) | ND | ND | 0.5 * (2) | ND | ND |
| D79A | 4600 (2) | >10000 | >10000 | 0.5 (2) | | 0% |
| N86A | 2950 (2) | >10000 | >10000 | 2.2 (2) | >1000 | 34% |
| F89A | 2.2 * (2) | ND | ND | 0.1 * (2) | ND | ND |
| N90A | 662 (2) | >10000 | >10000 | 2.4 (2) | >1000 | 56% |
| Y93A | >10000 | >10000 | >10000 | 1.9 (2) | >1000 | 55% |
| N97A | >10000 | >10000 | >10000 | 1.5 (2) | >1000 | 46% |
| W99A | UB * | | | UB | >1000 | 24% |
| Q109A | 290 (2) or 0.2* (2) | 12000 (2) | >10000 | 1.0 (2) | 6 (2) | 84% |
| F112A | 2.5 * (2) | 74 * (2) | 940 * (2) | 0.2 (2) | ND | ND |
| T115A | 0.2 * (2) | ND | ND | 0.5 (2) | ND | ND |
| Q166V | 120 (2) or 0.7* (2) | ND | ND | 3.2 (3) | 7 (2) | 83% |
| Y169A | 1160 (2) | >10000 | >10000 | 0.5 (2) | >1000 | 17% |
| S170A | 0.2 * (2) | ND | ND | 1.0 (2) | ND | ND |
| H198A | 754 (2) | >10000 | >10000 | 0.9 (2) | 87 (3) | 50% |
| Y206A | 1160 (2) | 5800 (2) | >10000 | 0.8 (3) | 35 (3) | 100% |
| Y266F | 1.5 * (2) | ND | ND | 2.0 (2) | 6 (2) | 100% |
| H267L | 2000 (2) | 2900 (2) | >10000 | 1.8 (2) | >1000 | 21% |
| H267F | 3500 (2) | >10000 | >10000 | 1.2 (2) | 16 (3) | 100% |
| F270A | 9300 (3) | >10000 | >10000 | 1.5 (2) | >1000 | 63% |
| S274A | 0.4 * (2) | ND | ND | 0.5 (2) | ND | ND |
| H282A | 0.3 * (2) | ND | ND | 0.3 * (2) | ND | ND |
| Q286A | 5200 (3) | >10000 | >10000 | 0.6 (2) | 340 (2) | 93% |
| Y289F | 0.2 * (2) | ND | ND | 300 * (2) | 34 (2) | 100% |
| F293A | 580 (2) | 5200 (2) | >10000 | 0.7 (2) | 65(3) | 73% |
| F270A-F293A | UB * | | | UB | >1000 | 20% |
| H267F-F293A | UB * | | | UB | >1000 | 20% |

[a] $K_i$ was determined from the inhibition of [$^3$H]SR48968 binding (unmarked entries) or [$^{125}$I]NKA (entries indicated by *). UB, undetectable binding of radioligand under the conditions of the assay. The number in parentheses is the number of independent experiments. [b] The $EC_{50}$ value was determined by functional assays such as those shown in Figure 5A. The $EC_{50}$ value for some mutants was not determined because of the lack of a clear maximal plateau at 10 $\mu$M NKA, the maximal concentration of peptide that can be used in these assays. The maximal response is determined in direct comparison to the wild-type NK2R (defined as 100%), keeping a constant cell number per assay. ND, not determined.

of NKA (N86A, N90A, and Y169A), suggesting that these modifications probably affect both agonist binding and the receptor–G protein interaction. Computer simulation has indicated that, all other parameters being equal, decreasing the affinity of G protein for the receptor can result in an $EC_{50}$ value higher than the $K_d$ value of an agonist (Kenakin & Morgan, 1989).

*Effects of Amino Acid Substitutions on Non-Peptide Antagonist Binding.* Previous experiments have demonstrated that several non-peptide NK1 antagonists interact with Gln-165, Ser-169, His-197, His-265, Tyr-272, and Tyr-287 of the NK1R, since substitution of any one of these residues resulted in a specific reduction in the affinity of one or more classes of NK1 antagonists (Cascieri et al., 1994; Fong et al., 1994; Gether et al., 1994; Huang et al., 1994a,b). To determine whether analogous residues in the NK2R are also required for the binding of the non-peptide NK2 antagonist SR48968, the counterparts in the NK2R (i.e., Gln-166, Ser-170, His-198, His-267, Ser-274, and Tyr-289) were mutated and their binding affinities for antagonists were determined. Q166V did not affect the binding affinity of NKA and slightly reduced the affinity of SR48968. Y289F did not affect the binding of NKA, while it substantially reduced the binding affinity of SR48968. S170A and S274A did not affect the affinity of either NKA or SR48968. In contrast, H198A and H267L substantially reduced the affinity of NKA without affecting that of SR48968 (Table 1). Therefore, the binding interactions utilized by SR48968 to bind to the NK2R do not appear to be identical to those involved in NK1 antagonist binding to the NK1R.

To further map the NK2 antagonist binding site, other nearby residues were selected for further mutagenesis studies. When Tyr-266 was substituted by Ala or Ser, no significant binding of [$^{125}$I]NKA or [$^3$H]SR48968 was detected. However, both Y266A and Y266S mutants were activated by NKA in functional assays (Figure 5). The binding affinity of SR48968 for the Y266S mutant, estimated from inhibition of NKA-elicited functional response, was substantially reduced compared to the wild-type receptor (Table 2). When Tyr-266 was substituted by Phe, essentially all parameters were identical to those of wild-type receptor.

When Phe-270 or Phe-293 was substituted with Ala, the binding affinity of SR48968 was not affected, while the affinity for NKA was reduced (Table 1). Because several studies have demonstrated interactions between NK1 antagonists and residues of helices 6 and 7 of the NK1R (Fong et al., 1994; Huang et al., 1994a) or have implicated helices 6 and 7 in the binding of NK2 antagonists (Gether et al., 1993), we further investigated the role of Phe-270 and Phe-293 in SR48968 binding by substituting both residues simultaneously. The double mutant F270A-F293A did not have a detectable level of [$^3$H]SR48968 binding, in contrast to the high-affinity binding of SR48968 displayed by either single residue mutant alone. However, this double mutant was active in the functional assay, although the dose–response curve for NKA was shifted to the far right (Table

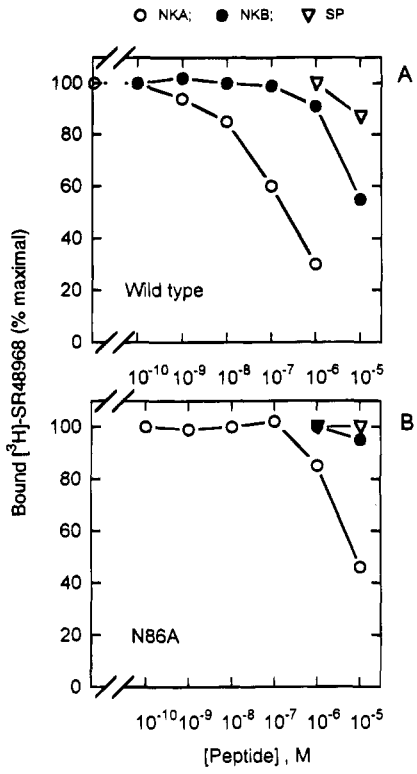


FIGURE 3: Inhibition of [$^3$H]SR48968 binding to the wild-type receptor (A) or the N86A mutant receptor (B) by NKA, NKB, or SP. Experimental conditions were similar to those in Figure 1B. The data shown are the average of at least two independent experiments.

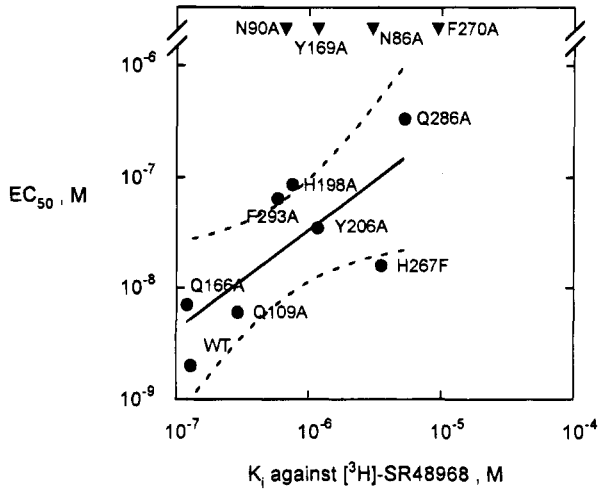


FIGURE 4: Comparison of NKA potency in stimulating PI hydrolysis ($EC_{50}$) and NKA binding affinity ($K_i$). Each point represents one receptor taken from Table 1. Receptors having undetectable binding of radioligand or having a $K_i$ value larger than 10 $\mu$M are not included in the graph. Triangles represent receptors whose $EC_{50}$ value is higher than 1 $\mu$M but cannot be precisely determined because of the lack of a clear plateau at 10 $\mu$M NKA. The dotted curves represent the 95% confidence limits of the linear regression through the data represented by closed circles (correlation coefficient = 0.78).

1). The substantially reduced potency of NKA in activating the F270A-F293A double mutant is consistent with the observation that both F270A and F293A mutants have reduced binding affinity for NKA. Another double mutant, H267F-F293A, had properties similar to those of the F270A-F293A double mutant (Table 1). Thus, with respect to SR48968 binding, simultaneously mutating two residues

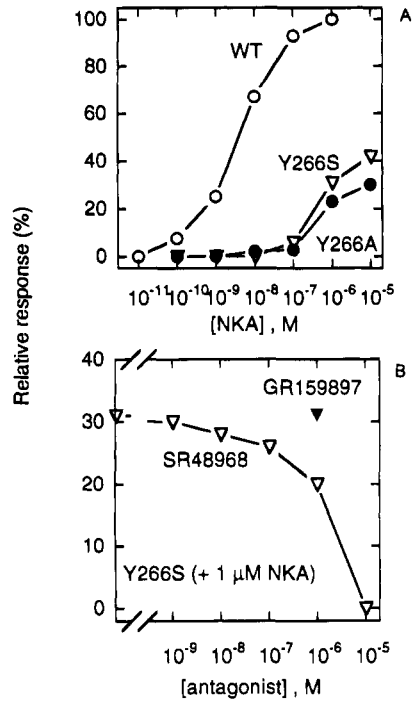


FIGURE 5: Stimulation of PI hydrolysis by NKA. (A) Dose-response curves of wild-type NK2R and the Y266A or Y266S mutant. All data are normalized to that of wild-type NK2R at 1 $\mu$M NKA. (B) Inhibition of NKA (1 $\mu$M) elicited response of the Y266S mutant by SR48968 or GR1598978. The data shown are the average of two independent experiments.

(Phe-270 plus Phe-293, or His-267 plus Phe-293) produced an effect that was more than the sum of those resulting from two point mutations.

In contrast to SR48968, another non-peptide NK2 antagonist GR159897 had reduced affinity for the H198A and F293A mutants (Table 3). Similar to SR48968, GR159897 affinity for the Y266S or Y289F mutant was reduced (Figure 5; Table 3).

*Inhibition of Receptor Activation by Non-Peptide Antagonist SR48968.* The initial mutational analysis of the NK2 receptor suggests that it is rare to identify a residue where substitution affects the binding of only SR48968 but not agonists. Only the Y289F substitution belongs to this category. This is in contrast to the NK1R where several residues required for antagonist binding but not agonist binding have been identified (Fong et al., 1994). To further investigate the relationship between the agonist binding site and the SR48968 binding site in the NK2R, the ability of SR48968 to inhibit the functional activation of wild-type NK2R was determined. As shown in Figure 6, SR48968 appeared to be a competitive antagonist of both NKA and NKA(4−10), a C-terminal fragment of NKA. For both peptide agonists, SR48968 increased the apparent $EC_{50}$ value without affecting the maximal response.

## DISCUSSION

Extensive mutational analysis of ligand binding sites in the NK1R has provided insights into the mechanism of peptide−receptor interaction and the crucial differences between peptide binding and small molecule binding (Fong & Strader, 1994; Huang et al., 1994a; Strader et al., 1994). Nonetheless, it is still unclear what molecular determinants are responsible for the subtype selectivity of the three

Table 2 : Effect of Substituting Tyr-266 or Tyr-289 on Agonist and Antagonist Binding Affinities (nM)

| | binding assay ($K_i$) [a] | | functional assay | | | |
|---|---|---|---|---|---|---|
| | | | inhibition | | | |
| receptor | NKA | SR48968 | $EC_{50}'$ of NKA | $IC_{50}'$ of SR48968 | @ [NKA] | $K_d$ of SR48968[b] |
| NK2R | 1.0 (3)* | 0.7 (5) | 2 (3) | 8 (2) | 100 | 0.2 |
| Y266A | UB* | UB | 300 (2) | ND | | ND |
| Y266S | UB* | UB | 300 (2) | 3400 (2) | 1000 | 500 |
| Y266F | 1.5 (2)* | 2.0 (2) | 6 (2) | ND | | ND |
| Y289F | 0.2 (2)* | 300 (2)* | 34 (3) | 5500 (2) | 100 | 2800 |

[a] Either [$^{125}$I]NKA was used to determine the $K_i$ values (entries marked by *) or [$^3$H]SR48968 was used to determine the $K_i$ values (unmarked). UB, undetectable binding of radioligand. ND, not determined. The number of independent experiments is shown in parentheses. [b] $K_d$ was calculated from the Gaddum equation, $K_d = IC_{50}'/(([NKA]/EC_{50}') - 1)$ (Lazareno & Birdsall, 1993).

Table 3 : Comparison of SR48968 Binding and GR159897 Binding

| | $K_i$, (nM)[a] | |
|---|---|---|
| | GR159897 | SR48968 |
| NK2R | 4 (2) | 0.7 (5) |
| Q166V | 4 (2) | 3.2 (3) |
| S170A | 4 (2) | 0.9 (2) |
| H198A | 406 (3) | 0.9 (2) |
| Y266S | >1000[b] | 500[b] |
| H267L | 10 (3) | 1.8 (2) |
| Y289F | >1000[c] | 300 (2)[c] |
| F293A | 87 (2) | 0.7 (2) |

[a] $K_i$ was derived from the inhibition of [$^3$H]SR48968 binding, except those marked by *b* or *c*. The number of independent experiments is shown in parentheses. [b] Apparent affinity was estimated in the functional assay (see Table 2). GR159,897 at 1 $\mu$M did not inhibit the functional response of Y266S elicited by 1 $\mu$M NKA. [c] $K_i$ was derived from the inhibition of [$^{125}$I]NKA binding.

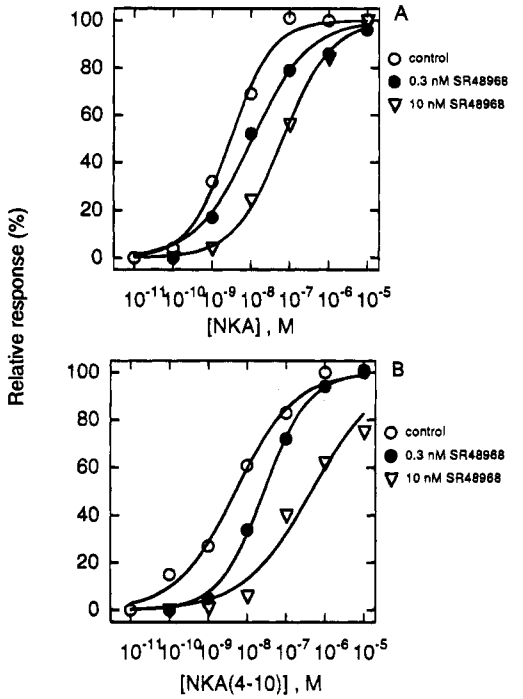


FIGURE 6: Dose−response curves of the wild-type NK2R in the absence or presence of SR48968. (A) Response elicited by NKA. (B) Response elicited by NKA(4−10). The data shown are the average of three independent experiments. All data are normalized to the response to 1 $\mu$M NKA.

neurokinin receptors. The present study was designed to investigate the peptide and non-peptide binding sites in the NK2R and to determine whether the NK1R and NK2R use a similar set of residues for ligand binding. As shown in

Table 4: Comparison of NK1R and NK2R Residues and Their Involvement in Peptide Binding[a]

| NK1R residue | involved in binding[b] | NK2R residue | involved in binding |
|---|---|---|---|
| Asn-23 | + | Thr-24 | + |
| Gln-24 | + | Ala-25 | ND |
| Phe-25 | + | Phe-26 | + |
| Asn-85 | + | Asn-86 | + |
| Asn-89 | + | Asn-90 | + |
| Tyr-92 | + | Tyr-93 | + |
| Asn-96 | + | Asn-97 | + |
| Trp-98 | + | Trp-99 | + |
| His-108 | + | Gln-109 | − |
| Gln-165 | + | Gln-166 | − |
| Tyr-168 | − | Tyr-169 | + |
| His-197 | − | His-198 | + |
| Tyr-205 | ± | Tyr-206 | + |
| His-265 | − | His-267 | + |
| Phe-268 | + | Phe-270 | + |
| Gln-284 | − | Gln-286 | + |
| Tyr-287 | + | Tyr-289 | − |
| Met-291 | + | Phe-293 | + |

[a] "+" indicates that conservative substitution or Ala substitution leads to reduced or increased peptide binding affinity. "−" indicates no effect with conservative substitution or Ala substitution. [b] Taken from Huang et al. (1994a).

Table 1, more than 10 residues in the extracellular and transmembrane regions of the NK2R have been identified that play a critical role in peptide agonist binding. Comparison with available data for similar mutants in the NK1R indicates that many conserved residues are commonly required for peptide binding in both the NK1R and the NK2R (Table 4). In addition, four conserved residues that are known not to be required for peptide binding to the NK1R are required for peptide binding to the NK2R (Tyr-168/169, His-197/198, His-265/267, and Gln-284/286, where the first number is the NK1R position and the second number is the NK2R position), and the opposite is found for three residues (His-108/Gln-109, Gln-165/166 and Tyr-287/289). On the basis of available data, all the commonly required residues are located in the E1, E2 and H2 regions, which is consistent with the hypothesis that the local conformations of the E1, E2, and H2 regions in both receptors are conserved with respect to peptide agonist binding. On the other hand, the determinants of differential peptide affinity are probably based on differences in either peptide conformation or the strength of interactions with receptor residues such as those identified here, or both.

For a given receptor, it is important to determine whether the different affinities for different peptides can be attributable to a simple address mechanism, i.e., whether one peptide recognizes one subset of receptor residues while another

peptide recognizes another subset. When the affinity of NKA for the NK2R is reduced by any one of the amino acid substitutions listed in Table 1, the binding affinities of NKB and SP are also reduced, suggesting that all these residues (either conserved or divergent) are required for binding of all three peptides. Similar results have also been obtained for the NK1R (Fong et al., 1992; Huang et al., 1994a). Thus, the address−message model needs to be modified to include the conformation factor. It remains a possibility that the N-terminal residues of neurokinin peptides may serve to stabilize the interaction between the conserved C-terminal half of the peptides and the receptor, rather than playing a direct role in receptor recognition. This hypothesis would suggest that the conformational compatibility between a ligand and a receptor is a primary basis of ligand selectivity in the neurokinin receptor family. A similar recognition mechanism has been proposed on the basis of structural analysis of drug−hemoglobin interactions, which demonstrate that small molecule binding is determined by the available van der Waals space of a potential binding pocket, and within that space, the bound molecule maximizes its electrostatic interactions with hemoglobin (Perutz et al., 1986).

An earlier report (Bhogal et al., 1994) indicated that the Q109H mutant displayed normal affinity for SR48968 but reduced affinity for NKA. This result has also been obtained in our laboratory (data not shown). However, the Q109A mutant was found to be identical to the wild-type receptor with respect to NKA and SR48968 binding (Table 1). Therefore, Gln-109 is apparently not required for peptide binding, although it may be located in the vicinity of the peptide binding site such that substitution with a His residue will be unfavorable for peptide binding. These data demonstrate the importance of analyzing several substitutions at the same position to differentiate whether a side chain is required for function (Ala substitution) or whether introduction of a different residue with other functionalities can produce a negative effect (Fong et al., 1995).

We have shown in previous studies that the H198A substitution does not affect the binding of SR48968 but reduces the affinity of NKA (Huang et al., 1994a). In the present report, we also demonstrated that the H198A mutant is active in stimulating the second messenger pathway, albeit with a higher $EC_{50}$ value compared to the wild-type receptor (Table 1). A recent paper indicated that no binding activity of [$^3$H]SR48968 was observed for the H198A mutant of the human NK2R (Bhogal et al., 1994). This discrepancy may perhaps be attributable to the differences in the protocols of the binding assay [rapid filtration of a cell suspension in the present study *versus* washing of a monolayer of cells in Bhogal et al. (1994)]. In the present study, we relied on both unaltered functional indices (antagonist binding affinity and agonist-stimulated second messenger production) and altered functional indices (lower affinity and lower potency for NKA) to reach the conclusion that His-198 and other residues listed in Table 1 play a specific role in peptide binding but not the binding of SR48968.

One important issue in the molecular definition of ligand−receptor interactions is whether mutational analysis of receptor function can explain the observed pharmacological properties. The data in Table 1 show that many substitutions affect the binding of either agonist or antagonist, but not both. Such an apparent lack of common residues for the binding of both agonist and antagonist should not be interpreted as evidence that a competitive antagonist and an agonist could bind to completely separate and non-overlapping sites on the receptor, where a binding site is the entire ligand−receptor interface. To avoid overinterpretation, it is necessary to consider the mutagenesis data in the context of a 3D receptor structure. If Gln-286 interacts directly with NKA and Tyr-289 interacts directly with SR48968 (Table 1), the proximity of these two residues clearly suggests an overlapping nature of agonist and antagonist binding sites. Furthermore, if the affinities of both antagonist and agonist are reduced for a mutant receptor such that radioligand binding cannot be measured directly, it would be difficult to confirm the ligand−receptor interactions using modified ligands or other approaches. Several substitutions fall into this category. The W99A substitution resulted in undetectable level of radioligand binding, and its $EC_{50}$ value could not be determined because a maximal plateau was not reached (Table 1). Whether Trp-99 participates in ligand binding or stabilizing receptor structure (Matsui et al., 1995) cannot be resolved due to the scarcity of experimental data. Substitution of Tyr-266 with Ala or Ser resulted in an undetectable level of agonist binding and antagonist binding. Fortunately, the Y266S mutant can be further characterized by functional assay (Figure 5). When agonist and antagonist affinities were estimated (Table 2), the data were consistent with the hypothesis that Tyr-266 is involved in the binding of both peptide and non-peptide ligands. Alternatively, it is possible that the substitution indirectly affects the binding of both ligands. If this is indeed an indirect effect, it is likely to be local because of the proximity of Tyr-266 to other residues whose substitution generates a more specific effect on peptide binding. Given these considerations, we postulate that the SR48968 binding site includes a subset of residues which also interact with peptide agonists. This hypothesis is consistent with the observation that SR48968 functions as a competitive antagonist of both NKA and a smaller analog, NKA(4−10) (Figure 6).

Interestingly, we observed that two double mutations (F270A-F293A and H267F-F293A) produce an effect larger than the sum of the two individual point mutations with respect to SR48968 binding (Table 1). One likely explanation would be that the flexibility of SR48968 enables it to interact with another nearby residue when a residue involved in antagonist binding is substituted. Alternatively, two residues may stabilize a third residue which interacts directly with SR48968. In either case, the affinity of the ligand may not change until both residues are substituted. On the basis of the present data, it is postulated that residues Tyr-266, His-267, Phe-270, Tyr-289, and Phe-293 comprise part of the SR48968 binding site. These studies also demonstrate the value of double mutations in studying the binding interactions of some ligands which may evade analysis based on single residue substitutions alone.

In summary, the present studies suggest that the peptide binding sites in the NK1R and NK2R share many common residues, especially in the E1, E2, and H2 regions. Residues (both conserved and divergent) in H4−H7 regions of the NK receptors seem to reflect the characteristics (e.g., local conformation) of a particular receptor subtype. For the NK2R, as well as the NK1R, differential peptide binding affinity is most likely based on conformational compatibility (or the bond distances between a ligand and a receptor) rather

than differential interactions with the N-terminal residues of peptides. These investigations provide a model of ligand−receptor interaction on which to base the design of direct structural analysis.

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